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(54) Title: IMPROVED BIOACTIVE WHEY PROTEIN HYDROLYSATE

(57) Abstract: The present invention relates to a process of preparing a whey protein hydrolysate from a WPI substrate having improved flavour, functionality and ACE-I inhibiting properties.



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IMPROVED BIOACTIVE WHEY PROTEIN HYDROLYSATE

TECHNICAL FIELD

5 This invention relates to a process for producing improved hydrolysed whey protein products which are substantially free of bitter flavours and which contain bioactive peptides. The products of the process have high digestibility and good organoleptic properties. The products have a bland taste and are free of soapy or brothy flavours. The improved hydrolysed whey protein products are useful sources of bioactive peptides for
10 incorporation into functional foods.

BACKGROUND ART

15 A number of food ingredients and foodstuffs have been produced from the hydrolysis of a protein source such as the milk proteins, casein and whey proteins.

Hydrolysed protein foodstuffs may have advantages over non-hydrolysed protein foodstuffs in a number of areas of health care. For example, it is known that enzymatically hydrolysed proteins are less allergenic. They are also more rapidly
20 digested and absorbed than whole proteins. Foodstuffs containing hydrolysed proteins are also useful in the alimentation of hospital patients with digestive diseases for example.

Hydrolysis of whey proteins and caseins is known to release bioactive peptides that can
25 exhibit a number of physiological effects (Maubois et al, 1991; EP 4745506). A number of publications describe such bioactive peptides, for example, ACE inhibiting peptides which have antihypertensive properties have been released through an enzymatic treatment of β -lactoglobulin and whey protein concentrates (Mullally et al, 1997). ACE inhibitory peptides are also found in sour milk and in hydrolysates of α_s and β casein (JP
30 4282400; Nakamura et al 1994, Yamamoto 1997).

EP 4745506 discloses the hydrolysis of the milk protein lactoferrin in whey to release lactoferricin which acts as an antimicrobial agent useful for treating diarrhoea, athlete's
35 foot, eye infections, mastitis etc in humans and animals.

However, the hydrolysis of most food proteins, especially the hydrolysis of whey and casein containing products, is known to generate bitterness. This causes palatability

problems particularly when attempting to formulate orally ingestible products incorporating milk protein hydrolysates as a source of bioactive peptides.

5 In the field of protein hydrolysis one or both of two approaches are commonly used for controlling or removing bitterness in protein hydrolysates to increase palatability of the products.

10 The extensive hydrolysis of the protein substrate is known to reduce bitterness in milk protein hydrolysates (EP 065663; EP 0117047; US 3970520). Less bitter products are produced relatively easily and cheaply in this way. However, extensive hydrolysis reduces the chain lengths of all peptides, including the bioactive peptides of interest. Extensive hydrolysis of the protein substrate destroys the functional and biological activity of the peptide of interest. In addition soapy and brothy off-flavours often develop, with the consequence that the palatability of the final product remains poor compared to the original bland tasting protein substrate. A final disadvantage is that for some hydrolysates the bitterness is only partially removed (Roy 1992 and 1997).

20 A second common method for the control of bitterness in protein hydrolysates is to use debittering enzymes, in particular those sourced from *Aspergillus oryzae*.

25 "Bitterness" generation in protein hydrolysis is thought to be due to the presence of large hydrophobic 'bitter' peptides. Debittering enzymes selectively hydrolyse bitter peptides present in the protein hydrolysates. A worker skilled in the art can - by the judicious selection of debittering enzymes and the conditions of treatment - effectively debitter milk protein hydrolysates leaving intact the particular bioactive peptides of interest. However, use of debittering enzymes makes the process more expensive, and preservation of some of the bioactive peptide is not easily or successfully achieved. A further disadvantage is that debittering enzymes treatments have a tendency to release free amino acids into the final product and, as a consequence, the hydrolysates develop unpleasant brothy or soapy flavours (Roy 1992 and 1997).

30 The various methods of debittering the protein hydrolysates result in additional process steps and add costs to the manufacture of the final product. In addition the final product also becomes overbalanced in its supply of free amino acids.

35 It would be most advantageous if a process for hydrolysing protein could be developed which releases bioactive peptides of interest and which limits the formation of bitter

peptides and free amino acids, thereby allowing the original bland taste of the milk protein substrates to be retained.

Some bioactive peptides - in particular the antihypertensive peptides - are relatively stable during protein hydrolysis and are released very early during the hydrolysis of the milk protein substrate as shown in Figure 1.

The bitter flavours of milk protein hydrolysates can be improved by adding sugars or by hydrolysing natural sugars, such as lactose, already present in the milk protein substrate (Bernal and Jelen, 1989). For example sour wheys and cheese wheys are made more palatable when they have been sweetened by β -galactosidase and lactase hydrolysis of lactose (FR 2309154; US 4358464; JP 8056568).

In order to achieve a high flavour acceptability for a hydrolysed protein product which contains bioactive peptides, precise control of hydrolysis is required to prevent bitterness occurring.

A common method of termination of hydrolysis is by deactivation of the enzymes, usually by thermal deactivation at high temperatures, typically $> 90-100^{\circ}\text{C}$ for an extended period of time. However, this method cannot be used to stop the hydrolysis of whey proteins as any intact unhydrolysed whey proteins remaining in the mixture would denature and precipitate making the final product less soluble and less acceptable for the use as a food ingredient.

Such a problem was overcome in WO 99/65326 which discloses a process of mild hydrolysis of sweet whey or sweet whey protein concentrate (WPC) to produce hydrolysates containing bioactive peptides having one or more of the following properties:

- antihypertensive ACE-I activity
- bifidus growth promoting activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste
- good organoleptic properties.

The present invention uses a different whey protein-containing substrate to that used in WO 99/65326 although a similar hydrolysing process is used. Surprisingly, the use of this different substrate results in a hydrolysate which shows dramatic improvements in

the above mentioned properties of the whey hydrolysates, particularly in the antihypertensive ACE-I activity, flavour and functionality of the product.

5 It is broadly to the process of hydrolysing a different whey protein containing substrate and the novel hydrolysate produced by this process that the present invention is directed.

SUMMARY OF THE INVENTION

10 Accordingly the invention may be said broadly to consist in a process for preparing an improved whey protein hydrolysate containing bioactive peptides comprising hydrolysing a whey protein isolate (WPI) with one or more enzymes characterised in that:

- i) the enzyme is a heat labile protease;
- 15 ii) the hydrolysis is conducted at a temperature of between about 30°C and 65°C at a pH of about 3.5 to about 9.0 when said enzyme is a neutral protease, at a pH of about 2.5 to about 6.0 where said enzyme is an acid protease; and at a pH of about 5.0 to about 10.0 where said enzyme is an alkaline protease;
- 20 iii) the hydrolysis is terminated when a degree of hydrolysis of no greater than about 10% has been reached;
- iv) the hydrolysis is terminated by deactivating said one or more enzymes; and
- v) the conditions for said step iv) are sufficiently mild to avoid substantial denaturation of peptides or residual proteins in said hydrolysate;

25 wherein the product produced by the process is highly soluble.

By WPI is meant a whey protein isolate produced by any method known in the art. Preferably the WPI is produced by a method of ion exchange from a whey protein concentrate, such as a cheese, acid, or lactic whey protein concentrate, as would be appreciated by a person skilled in the art.

30 By heat labile is meant that the enzyme is susceptible to irreversible deactivation at relatively moderate temperatures as would be appreciated by a person skilled in the art.

Preferably the enzyme is selected from the group consisting of Protease P6, Protease A, 35 Protease M, Peptidase, Neutrase, Validase, AFP 2000, and any other heat labile protease.

The enzyme hydrolysis step may be carried out under conditions which are suitable for the particular enzyme used as would be understood by a person skilled in the art.

The whey protein isolate (WPI) may be hydrolysed at a concentration in the range of from about 5-35% solids and the enzyme or enzyme mixtures may be added to give an enzyme to substrate ratio between about 0.01% and about 3% w/w total solids, preferably between about 0.01% and about 1.0% w/w total solids.

WPI treated with acid proteases may be hydrolysed at a pH of between about 2.5 and about 6.0, preferably between a pH of about 3.0 and about 5.0.

WPI treated with neutral proteases may be hydrolysed at a pH of between about 3.5 and about 9.0, preferably between a pH of about 6.0 and about 8.0.

WPI treated with alkaline proteases may be hydrolysed at a pH range of between about 5.0 and about 10.0, preferably between a pH of about 6.0 and about 8.0.

The protein hydrolysis may be carried out at a temperature range of from between about 30-65°C, preferably from about 50-60°C.

In one embodiment the one or more enzymes used to selectively hydrolyse the WPI may be immobilised on an inert support during said hydrolysis step ii) wherein said inert support is Roehm Eupergit, carrageenan particles, chitosan particles or any other suitable inert support material. This enzyme system may then be used in a stirred tank or fixed bed reactor or on a membrane or hollow fiber reactor to carry out the hydrolysis reaction.

The enzyme(s) used for hydrolysis may be cross linked to the said inert support prior to the hydrolysis reaction.

The hydrolysate of the present invention is referred to as a "mild" hydrolysate whereby the degree of hydrolysis, i.e. the percentage of peptide bonds cleaved by enzymic action is less than about 10%. Thus, although the hydrolysate may still contain large peptide chains that may be slightly denatured, the final hydrolysate product is highly soluble.

The degree of hydrolysis of the WPI substrate is preferably from about 3% to about 10%, most preferably from about 3% to about 5% before the hydrolysis is terminated.

Hydrolysis is determined by the enzyme deactivating step iv). Preferably, enzyme deactivation comprises heat deactivation.

The heat deactivation may comprise heating said hydrolysate for up to about ten seconds to a temperature of up to about 100°C.

When the hydrolysis is conducted at a temperature of below 65°C, the heat deactivating step is conducted at about 65°C to about 70°C for from about 10 seconds to about 15 minutes.

When the hydrolysis is conducted at a temperature of below 60°C, the heat deactivating step is conducted at about 60° to about 65°C for from about 10 seconds up to about 30 minutes.

Alternatively, the enzyme deactivating step iv) comprises altering the pH of said whey protein-containing substrate to a pH at which said protease is not active.

According to one option, depending on the enzyme(s) used, the enzyme or enzyme mixture may also be deactivated by the evaporation and drying procedures.

According to another option the enzyme or enzyme mixture may also be deactivated with or without a prior pH change.

Alternatively, the enzymes may be deactivated by simply removing them from the reaction mixture. For example, when the one or more enzymes used to selectively hydrolyse the WPI are immobilised on an inert support, such as by cross-linking to said inert support prior to the hydrolysis reaction, they may be subsequently separated out of the hydrolysis reaction by membrane filtration in order to achieve deactivation.

Alternatively, the enzyme(s) may be separated out of the hydrolysis mixture with the use of an ultrafiltration membrane with a nominal molecular weight cutoff in the range of about 10-500 kDa, preferably about 10-200 kDa, once hydrolysis is complete.

In a preferred embodiment, termination of the hydrolysis is achieved by deactivating the one or more whey protein hydrolysis enzymes by firstly changing the pH of the reaction mixture to a pH in which the enzyme(s) is either inactive or less active, and/or heating the reaction mixture to a comparatively mild temperature using a heat exchanger to denature the enzyme but not the intact whey proteins in the substrate. A suitable temperature range which would denature the enzymes is in the order of about 55-70°C, preferably about 65°C.

After hydrolysis and optional deactivation or removal of enzymes, the hydrolysate may optionally be subjected to reverse osmosis under conditions whereby salt and water are removed from the hydrolysate. The purified desalted hydrolysate comprising whey proteins, polypeptides and bioactive peptides is then recovered.

5

Optionally the hydrolysed WPI containing the bioactive peptide fraction can be purified with a UF membrane of about 5-200 kDa cut off, preferably about 10-50 kDa cut off. The bioactive peptides, and other peptides are recovered in the permeate.

10 According to another option ion exchange or hydrophobic adsorption or hydrophobic interaction chromatography or combinations of these processes may be used to recover the hydrolysed bioactive fraction from the hydrolysates in an enriched form.

15 In another embodiment the invention consists in a whey protein hydrolysate containing one or more bioactive peptides selected from the group consisting of SAP (SEQ ID NO: 1), MKG (SEQ ID NO: 2), ALPMH (SEQ ID NO: 3), LIVTQ (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

20 Preferably the whey protein hydrolysate comprises at least one bioactive peptide selected from the group consisting of LIVTQ (SEQ ID NO: 1), MKG (SEQ ID NO: 2) and ALPMH (SEQ ID NO: 3), in combination with at least one bioactive peptide selected from the group comprising SAP (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID
25 NO: 8).

According to a further aspect the present invention provides a pharmaceutical composition comprising one or more of the bioactive peptides produced in the process of the invention together with a pharmaceutically acceptable carrier. Preferably said
30 pharmaceutical composition comprising at least the bioactive peptide MKG (SEQ ID NO: 2) together with a pharmaceutical acceptable carrier.

According to a still further aspect, the present invention provides a method of treating or preventing hypertension in a mammal comprising administering an effective amount
35 of a bioactive peptide produced by hydrolysing WPI according to the process of the invention to a mammal in need thereof.

According to a still further aspect, the present invention provides a use of one or more bioactive peptides produced by the process of the invention in the manufacture of a medicament for treating or preventing hypertension in a patient in need of such treatment. Preferably said bioactive peptide is MKG (SEQ ID NO: 2).

5

The present invention further provides a non-bitter, highly soluble WPI hydrolysate product prepared by the process of the invention, wherein the degree of hydrolysis of WPI is about 3% to about 10%. The mean particle size of whey proteins in the product may be less than about 30 microns, preferably less than about 3 microns.

10

In a further embodiment the invention provides a food product containing a WPI hydrolysate of the invention.

15

The invention further comprises a method of reducing systolic blood pressure in a subject, wherein said method comprises administering an effective amount of the novel WPI hydrolysate or food product containing said hydrolysate of the invention to a subject in need thereof.

20

The invention further comprises one or more peptides selected from the group comprising SAP (SEQ ID NO: 1), MKG (SEQ ID NO: 2), ALPMH (SEQ ID NO: 3), LIVTQ (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

25

The invention further comprises at least one bioactive peptide selected from the group consisting of LIVTQ (SEQ ID NO: 1), MKG (SEQ ID NO: 2) and ALPMH (SEQ ID NO: 3), in combination with at least one bioactive peptide selected from the group comprising SAP (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

30

The hydrolysed WPI product of the invention has one or more of the following features:

35

- antihypertensive ACE-I activity
- probiotic growth promoting activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste
- good organoleptic properties
- high solubility
- very good foaming properties

- very good gelling properties
- improved heat stability

The application of the mild hydrolysis technology to the substrate of whey protein isolates (WPI) revealed some dramatic improvements of the final product in comparison to sweet whey protein concentrate (WPC) as a whey-protein containing substrate as disclosed in WO 99/65326 as follows:

- Solubility

Although the WPI still denatures slightly on the heat conditions to stop the protease reaction, it does not produce any insoluble material. The solubility remains around 96% which is greater than the corresponding WPC hydrolysate.

- Heat stability

The hydrolysed WPI was significantly more heat stable than the hydrolysed WPC. After 120°C for 10 min @ 5% TS the solubility remains 95%.

- Appearance

Proper selection of reaction conditions can determine whether the final hydrolysate will look white or clear in solution. Example 1, below, produces an opaque product, whilst Examples 2 and 3 (below) result in clear product in solution at neutral pH. Hydrolysates of WPC were all substantially white in appearance.

- Foaming ability and stability

The hydrolysed WPI shows about double the foaming ability and about four times the foam stability of non-hydrolysed WPI. This makes the product very suitable as an ingredient for yoghurt and dessert applications. The hydrolysed WPI also showed improved foaming ability and stability compared to a WPC hydrolysate.

- Gel strength

The mildly hydrolysed WPI shows a markedly increased gel strength compared to non-hydrolysed WPI. This makes the product very suitable as an ingredient for yoghurt and dessert applications. The hydrolysed WPI also showed improved gel strength compared to a WPC hydrolysate.

- Flavour

The hydrolysed WPI shows significantly less bitterness compared to mildly hydrolysed WPC products. The bitterness shows no tendency to increase over time of hydrolysis, making the control of the process much easier in comparison to WPC hydrolysis.

5

- ACE-I activity in vitro

The hydrolysed WPI shows about double the ACE-I activity in vitro of mildly hydrolysed WPC, given comparable reaction conditions and enzyme addition.

10

- Acceleration of probiotic fermentation

The hydrolysed WPI shows an acceleration in the rate of probiotic yoghurt fermentation time of about 40% at an addition rate of 1.5%.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

15

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described with reference to the accompanying drawings in which:

20

Figure 1 is a plot of bitterness and bioactivity on the ordinant against the degree of hydrolysis on the abscissa. The 'opportunity window' of obtaining a product according to the present invention containing bioactive peptides and having acceptable flavours before the hydrolysis reaction produces bitter peptides is between the lines x_1 and x_2 .

25

Figure 2 shows the Acute Short Term Effect of Systolic Blood Pressure of dosing mature spontaneously hypertensive rates (SHRs) with 6.0g WPC hydrolysate/kg body weight.

30

Figure 3 shows the Acute Short Term Effect of Systolic Blood Pressure of dosing mature spontaneously hypertensive rates (SHRs) with 3.6g WPI hydrolysate/kg body weight.

35

Figure 4 shows the Acute Short Term Effect of Systolic Blood Pressure of dosing mature spontaneous hypertensive rates (SHRs) with 165mg peptide MKG (SEQ ID NO: 2)/kg body weight.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention provides a process for producing a hydrolysed WPI product containing bioactive peptides, whereby the hydrolysis is carried out under a high degree of control to prevent undesirable flavours developing during hydrolysis (e.g. bitter, soapy and brothy). The hydrolysis is terminated within the "opportunity window", i.e. before the emergence of substantial bitterness - as shown in Figure 1 - to provide hydrolysates having good organoleptic properties and maximum bioactive peptides. In Figure 1 the degree of hydrolysis is represented qualitatively on the x axis. The window of opportunity is between the points x_1 and x_2 which will vary depending on the enzyme which is used. The optimum conditions sought are a maximum bioactivity with an acceptable level of bitterness.

In particularly preferred embodiments of the process of the invention, the enzyme which hydrolyses the WPI is heat labile and is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined) and the hydrolysis of the WPI is terminated by heat treatment for a short time at a high temperature (about 85-100°C for about 1-10 seconds). The applicants have surprisingly found that the above enzymes (1) are able to produce a whey protein hydrolysate containing a good level of bioactive peptides, and (2) can be inactivated by a short time, high temperature treatment which causes only partial denaturation of the whey proteins in the hydrolysate, and surprisingly improves the organoleptic properties of the whey proteins, in terms of providing a product which is substantially white or clear in appearance.

The present invention is now exemplified by the following examples using ALACEN™ 895 or ALACEN™ 894 (whey protein isolates, commercially available from NZDB), the product specifications for which are attached in Appendix I:

Example 1

Pilot plant production of WPI mild hydrolysate

5

Whey protein isolate produced by cation ion exchange technology (ALACEN™ 895) with a protein content $\geq 90\%$ w/w was reconstituted at 20% total solids in water (50°C). Reconstituted ALACEN™ 895 was transferred to a 150L tank at 50°C. Water (50°C) was added to the tank to make final total solids of 4%. The solution was stirred and

10

Neutrase (E:S 0.9%) was added.

Two hours after enzyme addition the first hydrolysate was pumped through the UHT plant. Enzyme inactivation was achieved using direct steam injection to heat the hydrolysate to 88°C and the hydrolysate was held at this temperature for 1.5 seconds.

15

The hydrolysate was flash cooled and passed through shell and tube heat exchanges to cool to ambient temperature.

The hydrolysate was subsequently evaporated and dried.

20

A hydrolysate made following the process of Example 1 had the following features:

Solubility: 95%

Heat stability: 120°C for 10 min @ 5% TS solubility 95%

ACE-I in vitro activity: 289 mg/L IC50

25

Foaming: Markedly increased over non-hydrolysed WPI

Flavour: Markedly improved over WPC based mild hydrolysates

Appearance: opaque white, particle size $\sim 0.1 \mu\text{m}$

30

The solubility, heat stability, foaming, and appearance of the WPI hydrolysate were measured by standard methods familiar to those skilled in the art. The solubility of 5% total solids (TS) solution was determined by centrifugation at 3000 g for 10mins (at room temperature). The heat stability of 5% TS solutions was determined by

heating to 120°C for 10mins, quick cooling, then centrifugation at 700 g for 10mins.

The TS contained in the supernatant and the original solution was determined. The

35

solubility was defined as the TS(supernatant)/TS(original solution). Foaming of 10%

TS solutions at pH 7.0 was determined by whisking with a Hobart mixer (Model N-50G, Hobart Corporation) for 15 mins. The percentage overrun was used to compare the sample as would be understood by a person skill in the art. The appearance of 5% TS was determined by visual observation and the particle size was measured using a
5 Malvern MasterSizer (Model MSE00SM, Malvern Instruments Ltd).

ACE-I activity (*in vitro*) in the dried product was determined using FAPGG as a substrate (Product 305-10 ex Sigma Chemical Corporation, St Louis, MO, USA) according of the method of D W Cushman & H S Cheung (1971). ACE-I activities
10 are expressed as the amount of material (mg/L) needed to reduce the activity of the ACE-I enzyme by 50%.

The flavour was assessed subjectively with reference to the bitterness and astringency of the hydrolysates, in particular, the sensory profile was assessed by a formal sensory
15 panel. 5% w/w samples were tasted at 24°C using multi-dimensional scaling. Samples were evaluated and scored on a 150mm anchored line (absent (0) to intense (150)).

Example 2

20 The hydrolysis reaction was repeated as outlined above for Example 1 (10% total solids, E:S 0.9%, 150L). After inactivation the hydrolysate was immediately evaporated and dried

25 A hydrolysate made by this method had the following features which were measured as disclosed above for Example 1:

Solubility: 97%

Heat stability: 120°C for 10 min @ 5% TS solubility 96%

30 ACE-I in vitro activity: 503 mg/L IC50

Foaming: Markedly increased over non-hydrolysed WPI

Gelling: Markedly increased over non-hydrolysed WPI

Flavour: Markedly improved over WPC based mild hydrolysates

Appearance: clear yellowish

Example 3

- 5 The hydrolysis reaction of Example 1 was repeated as outlined above (4% total solids, E:S 0.9%, 150 L). Four hours after enzyme addition the first hydrolysate was pumped through the UHT plant using the same conditions as in Example 1.

- 10 A hydrolysate made by this method had the following features which were measured as disclosed above for Example 1:

ACE-I in vitro activity: 230 mg/L IC50

Foaming: Markedly increased over non-hydrolysed WPI

Gelling: Markedly increased over non-hydrolysed WPI

- 15 Flavour: Markedly improved over WPC based mild hydrolysates

Appearance: clear yellowish

Example 4

- 20 A 2% solution of ALACEN™ 894 (micro-filtered WPI) was altered to pH 3.0 before undergoing ultrafiltration at 10°C with a 3,000 Dalton nominal molecular weight cutoff membrane (CDUF001LB, Millipore Corporation, Bedford). The pH of the retentate was altered to 7.0 and diluted to 2% total solids before ultrafiltration at 10°C with the same membrane used previously. The total solids of the retentate were adjusted to 5.0%
25 before being hydrolysed with E:S 0.9% w/w Neutrase (Novo Nordisk, Denmark) at 50°C. After 4h the sample was inactivated at 88°C for 3 seconds and subsequently freeze-dried. The ACE-I activity was determined to be 227 mg/L.

Example 5

Mildly hydrolysed WPI from Example 3 was shown to promote the growth of a probiotic microorganism when added to half fat milk at an addition rate of 1.5%. The milk was heat treated @ 90°C for 10 minutes. After cooling to 37°C the milk was inoculated with 0.1% *S. thermophilus* and 2% *L. rhamnosus* HN001(DR20™). The fermentation time of the control was 20h to reach the required pH of 4.4. In contrast, the WPI hydrolysate addition reduced the fermentation time to 13h.

Example 6

Identification of ACE-inhibitor peptides in WPI hydrolysates

The objective of this example was to isolate and identify ACE-inhibitor peptides present in the WPI hydrolysates

Methodology

- The starting material for ACE-I peptide isolation was a UF permeate (10 kDa) obtained from the original hydrolysate of Example 1;
- Peptides present in the hydrolysate were separated using reverse-phase HPLC;
- Purified peptides were assayed individually for ACE-inhibitor activity as described in Example 1;
- The amino acid sequence of each active peptide was identified by a combination of mass spectrometry and N-terminal sequence analysis;
- The origin of the active peptides was determined by comparing their sequences with the known sequences of the milk proteins.

The peptides, their origins, activities and known similarities are set out in Table 1 below:

TABLE 1: ACE-inhibitor peptides isolated from a WPI hydrolysate

5

Peptide	ACE-inhibitor sequence	Origin	¹ Activity (IC ₅₀)
1	SAP (SEQ ID NO: 1)	β-LG(36-38)	22
2	² MKG (SEQ ID NO: 2)	β-LG(7-9)	23
3	² ALPMH (SEQ ID NO: 3)	β-LG(142-146)	23
4	² LIVTQ (SEQ ID NO: 4)	β-LG(1-5)	19
5	VSLPEW (SEQ ID NO: 5)	α-LA(21-26)	8
6	³ INYWL (SEQ ID NO: 6) ³ LKPTPEGDLEIL (SEQ ID NO: 7)	α-LA(101-105) β-LG(46-57) }	11
7	LKGYGGVSLPEW (SEQ ID NO: 8)	α-LA(15-26)	7

¹mg/L²also detected in WPC hydrolysates of WO 99/65326.10 ³two peptides were present in the sample; one or both may be active peptides(s)

- Three of the active peptides from the WPI hydrolysate were previously identified in the WPC hydrolysate i.e. β-LG(7-9), β-LG(142-146) and β-LG(1-5).

15 **Example 7**

Sensory Comparison of mildly hydrolysed WPI compared to mildly hydrolysed WPC and unhydrolysed WPI

20 WPI was hydrolysed as set out above in Example 1.

WPC was hydrolysed as set out in Example 1 of WO 99/65326. (ALACENTM 392, 10%, hydrolysed and inactivated after 2 hours).

- 5 ALATALTM 819 is a commercial product of the New Zealand Dairy Board; the summary of the product specification of which is attached in Appendix I.

A taste panel scored the bitterness and astringency of the products scored on a 150mm anchored line (absent (0) to intense (150)).

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Degree of Hydrolysis was determined using the Modified O-phthaldialdehyde (MOPA) method as described by Frister *et al.* (1988).

15 **Table 2 Mean score (/150) for WPI and hydrolysate samples evaluated by a formal sensory panel, degree of hydrolysis, and ACE-I activity of standard WPI and hydrolysed whey protein.**

Product tested	Sensory Evaluation		Degree of Hydrolysis (%)	ACE-I activity (mg/L)
	Bitter	Astringent		
ALACEN TM 895 (WPI)	36.2	24	0	~2000
Mildly hydrolysed WPC	78	67.1	4.5	440
Mildly hydrolysed WPI	55.1	41.6	4.3	230
ALATAL TM 819	93.1	53.3	10	-

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Both mildly hydrolysed WPI and mildly hydrolysed WPC were more bitter and astringent than the non-hydrolysed WPI product as expected. However, these products were significantly less bitter and astringent than a more vigorously hydrolysed ALATALTM 819 (a product of the New Zealand Dairy Board, NZ).

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Mildly hydrolysed WPI products gave more acceptable flavoured products and had significantly higher ACE-I activity, compared to the mildly hydrolysed WPC (described in WO 99/65326). The results also showed that the mildly hydrolysed WPI was significantly less bitter and astringent than the equivalent WPC product, although the degree of hydrolysis for both was similar. As well as having a more acceptable flavour

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profile, the ACE-I activity of the mildly hydrolysed WPI was almost twice that of the mildly hydrolysed WPC.

Although further hydrolysis of WPC may result in an increased ACE-I activity, a significant decrease in the flavour profile compared to the mildly hydrolysed WPI is observed, compromising the flavour profile due to increased bitterness and astringency.

Example 8

10 Blood Pressure Measurement

Blood pressure was measured using a tail cuff monitor and a purpose designed apparatus for measuring the blood pressure of small animals [IITC Inc, 239 Victory Blvd., Woodland Hills, CA 91367, USA]. Each time point was the average of 3-5 readings taken within approximately 5-10 minutes.

The Animal Strain

Spontaneously hypertensive rats (SHRs) were sourced from the Animal Resource Centre, Western Australia.

Sample Dosage

The test substances were given to each rat on a per kg body weight basis unless otherwise stated. The individual dose for each animal was incorporated into a flavoured jelly, which was readily consumed by the animals.

The three samples evaluated for short term blood pressure response were a mildly hydrolysed whey protein concentrate (Figure 2), a mildly hydrolysed whey protein isolate (see Figure 3) and a synthesised peptide from Table 1, namely MKG (SEQ ID NO: 2) (see Figure 4). The WPC was prepared according to the methodology set out in example 1 of WO 99/65326.

Acute or short term effects of agents were determined by treating adult SHRs with the agent and then studying their blood pressure responses over the following hours. The study detects if an agent lowers blood pressure that is already elevated.

Results

Short term dosing of SHR rats with 3.6g/kg body weight of WPI hydrolysate (Figure 3) resulted in a significant reduction in blood pressure compared to control rats (p=0.0378). The reduction in blood pressure was similar to that achieved in rats dosed with 6.0g/kg body weight WPC hydrolysate (Figure 2). Thus, the WPI hydrolysate appears to be more active than the comparative WPC hydrolysate so that approximately 50% less of the WPI hydrolysate is required to give a similar decrease in blood pressure (systolic) in mature SHR rats.

Short term dosing of SHR rats with 165mg/kg body weight peptide MKG (Figure 4) showed a much greater reduction in blood pressure compared to control rats (p=0.0021 after 4 hours; p<0.0001 after 8 hours) as well as compared to the mild WPI hydrolysate and mild WPC hydrolysate.

Conclusions

The process of mildly hydrolysing WPI according to the present invention provides useful WPI hydrolysates which show significant functional improvement over the WPC hydrolysates of WO 99/65326. In particular, the present WPI hydrolysates comprise more active peptides with significantly less WPI hydrolysate required to decrease blood pressure in vivo to the same degree as a WPC hydrolysate. WPI can be hydrolysed for longer without losing acceptable flavour characteristics and whilst maintaining the increased ACE-I activity. Thus, overall, the process of the present invention provides a bioactive whey protein hydrolysate which is improved in flavour, ACE-I activity and functionality compared to that of WO 99/65326.

INDUSTRIAL APPLICATION

The process of the present invention is useful to produce the novel whey protein hydrolysate which has surprising beneficial properties and can be used as a food or medicine as an anti-hypertensive.

It will be appreciated that it is not intended to limit the invention to the aforementioned examples only, many variations, such as might readily occur to a person skilled in the art, being possible, without departing from the scope of the appended claims.

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SEQUENCE LISTING

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<150> NZ 506866
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35

WHAT WE CLAIM IS:

1. A process for preparing an improved whey protein hydrolysate containing bioactive peptides comprising hydrolysing a whey protein isolate (WPI) with one or more enzymes characterised in that:

- i) the enzyme is a heat labile protease;
- ii) the hydrolysis is conducted at a temperature of between about 30°C and 65°C at a pH of about 3.5 to about 9.0 when said enzyme is a neutral protease, at a pH of about 2.5 to about 6.0 where said enzyme is an acid protease; and at a pH of about 5.0 to about 10.0 where said enzyme is an alkaline protease;
- iii) the hydrolysis is terminated when a degree of hydrolysis of no greater than about 10% has been reached;
- iv) the hydrolysis is terminated by deactivating said one or more enzymes; and
- v) the conditions for said step iv) are sufficiently mild to avoid substantial denaturation of peptides or residual proteins in said hydrolysate;

wherein the production of the process is highly soluble.

2. A process according to claim 1, wherein the enzyme is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase, AFP 2000, and any other heat labile protease.

3. A process as claimed in claim 1 or 2, wherein said enzyme deactivating step iv) comprises heat deactivation.

4. A process as claimed in claim 3, wherein said heat deactivation comprises heating said hydrolysate for up to ten seconds to a temperature up to about 100°C.

5. A process as claimed in claim 3, wherein, when said hydrolysis is conducted at a temperature of below about 65°C, said heat deactivating step is conducted at about 65°C to about 70°C for from about 10 seconds to about 15 minutes.

6. A process as claimed in claim 3, wherein, when said hydrolysis is conducted at a temperature below about 60°C, said heat deactivating step is conducted at about 60°C to about 65°C for from about 10 seconds up to about 30 minutes.

7. A process as claimed in claim 1 or claim 2, wherein said enzyme deactivating step comprises altering the pH of said whey protein-containing substrate to a pH at which said protease is not active.

8. A process as claimed in claim 7, wherein said enzyme deactivating step includes heat deactivation as claimed in any one of claims 3 to 6.

9. A process as claimed in claim 1 or claim 2, wherein said enzyme deactivating step iv) comprises subjecting said hydrolysate to ultrafiltration with an ultrafiltration membrane having a nominal molecular weight cutoff in the range of about 10-500 kDa.

10. A process as claimed in claim 9, wherein said ultrafiltration membrane has a nominal molecular weight cut off in the range of about 10-200 kDa.

11. A process as claimed in any one of the preceding claims, wherein said enzyme is immobilised on an inert support during said hydrolysis step ii).

12. A process as claimed in claim 11, wherein said inert support is Roehm Eupergit, carrageenan particles, chitosan particles or any other suitable inert support material.

13. A process as claimed in any one of the preceding claims, wherein the degree of hydrolysis is from about 3% to about 10%.

14. A process as claimed in claim 13, wherein the degree of hydrolysis is from about 3% to about 5%.

15. A process as claimed in any one of the preceding claims, wherein the whey protein hydrolysate so produced comprises one or more bioactive peptides selected from the group consisting of SAP (SEQ ID NO: 1), MKG (SEQ ID NO: 2), ALPMH (SEQ ID NO: 3), LIVTQ (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

16. A process as claimed in any one of the preceding claims, wherein the whey protein hydrolysate so prepared comprises at least one bioactive peptide selected from the group consisting of LIVTQ (SEQ ID NO: 1), MKG (SEQ ID NO: 2) and ALPMH (SEQ ID NO: 3), in combination with at least one bioactive peptide selected from the group comprising SAP (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

17. A pharmaceutical composition comprising one or more of the bioactive peptides produced in the process of any one of claims 1 to 16 together with a pharmaceutically acceptable carrier.

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18. A pharmaceutical composition as claimed in claim 17, comprising the bioactive peptide MKG (SEQ ID NO: 2) together with a pharmaceutical acceptable carrier.

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19. A method of treating or preventing hypertension in a mammal, said method comprising administering an effective amount of a bioactive peptide produced by hydrolysing WPI according to the process of any one of claims 1 to 16 to a mammal in need thereof.

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20. A use of one or more bioactive peptides produced by the process of any one of claims 1 to 16 in the manufacture of a medicament for treating or preventing hypertension in a patient in need of such treatment.

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21. A use as claimed in claim 19, wherein said bioactive peptide is MKG (SEQ ID NO: 2).

22. A non-bitter, highly soluble WPI hydrolysate product containing bioactive peptides, prepared by the process of any one of claims 1 to 16.

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23. A product as claimed in claim 22, wherein the degree of hydrolysis of the WPI is about 3% to about 5%.

24. A product as claimed in claim 23, wherein the main particle size of whey proteins in the product is less than about 30 microns.

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25. A product as claimed in claim 24, wherein the main particle size is less than about 3 microns.

26. A product as claimed in any one of claims 22 to 25, which is substantially clear or white in solution.

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27. A product as claimed in any one of claims 22 to 26, wherein one or more of said bioactive peptides is selected from the group consisting of SAP (SEQ ID NO: 1), MKG (SEQ ID NO: 2), ALPMH (SEQ ID NO: 3), LIVTQ (SEQ ID NO: 4), VSLPEW (SEQ

ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

28. A product as claimed in any one of claims 22 to 26, comprising at least one bioactive peptide selected from the group consisting of LIVTQ (SEQ ID NO: 1), MKG (SEQ ID NO: 2) and ALPMH (SEQ ID NO: 3), in combination with at least one bioactive peptide selected from the group comprising SAP (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

29. A food product containing a WPI hydrolysate product as claimed in any one of claims 22 to 28.

30. A method of reducing systolic blood pressure in a subject comprising administering an effective amount of a WPI hydrolysate as claimed in any one of claims 22 to 28 or food product containing said hydrolysate as claimed in claim 29 to a patient in need thereof.

31. A use of a product as claimed in any one of claims 22 to 28 in the manufacture of a medicament for treating or preventing hypertension in a patient in need thereof.

32. A pharmaceutical composition comprising the product of any one of claims 22 to 28 together with a pharmaceutically acceptable carrier.

33. Any one or any combination of two or more peptides selected from the group comprising SAP (SEQ ID NO: 1), MKG (SEQ ID NO: 2), ALPMH (SEQ ID NO: 3), LIVTQ (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

34. Any one bioactive peptide selected from the group consisting of LIVTQ (SEQ ID NO: 1), MKG (SEQ ID NO: 2) and ALPMH (SEQ ID NO: 3), in combination with at least one bioactive peptide selected from the group comprising SAP (SEQ ID NO: 4), VSLPEW (SEQ ID NO: 5), INYWL (SEQ ID NO: 6), LKPTPEGDLEIL (SEQ ID NO: 7) and LKGYGGVSLPEW (SEQ ID NO: 8).

APPENDIX I

ALACEN™ 895

Whey Protein Isolate

P613.04.0898

ALACEN 895 is a soluble, 93% milk protein product with low fat, lactose and cholesterol giving nutritional benefits as well as providing enhanced functional and flavour properties.

Functionally, ALACEN 895 is a specialised protein providing acid/heat stability, solution clarity and aeration stability.

PRODUCT CHARACTERISTICS

- Excellent whipping capability
- Clean flavour
- Low lactose
- Low fat and low cholesterol content
- Acid/heat stability
- Soluble over a wide pH range
- Excellent nutritional properties

SUGGESTED USES

ALACEN 895 is particularly useful for applications which are flavour sensitive and require a nutritional protein product.

- High protein beverages
- Low fat and low lactose foods
- Meringue and whipped toppings
- Protein fortified nutritional foods

TYPICAL COMPOSITION

Energy	1616 kJ/100g
Protein (N x 6.38) is	as 93.1%
dry basis	96.6%
Fat	0.6%
Moisture	3.6%
Ash	1.6%
Lactose (by difference)	1.1%

TYPICAL CHEMICAL ANALYSES

pH (5% at 20°C)	6.8
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PHYSICAL PROPERTIES

Colour	Cream
Flavour	Clean
Sediment (50 g)	Disc A

TYPICAL MICROBIOLOGICAL

Aerobic Plate Count (cfu/g)	< 30,000
Coliforms (1g)	Negative
Yeasts and moulds (cfu/g)	< 10
Coagulase Positive Staphylococci (1g)	Negative
Salmonella (750g)	Negative

TYPICAL MINERAL COMPOSITION

Sodium	484 mg/100g
Potassium	80.1 mg/100g
Chloride	21 mg/100g
Calcium	106 mg/100g
Phosphorous	52.5 mg/100g

PACKAGING

The standard pack is a multi-wall bag which incorporates a moisture barrier and contains the products within an inner polythene liner. No staples or metallic fasteners are used.

Net weight	25.0kg
Gross weight	25.5kg

STORAGE AND HANDLING

ALACEN 895 should be kept in a cool, dry ventilated place. Temperatures below 25°C, relative humidities below 65% and an odour free environment will extend storage life. Packages should not be in contact with walls or floors. Stock should be used in rotation, preferably within six months of delivery.

QUALITY ASSURANCE

Strict quality control procedures are enforced during manufacture. The manufacturing environment is also subject to regular monitoring and control.

Final product is sampled and tested for chemical, sensory and microbial parameters using internationally recognised procedures.

During storage and shipment, precautions are taken to ensure that product quality is maintained. Each package is identified, enabling traceback

ALACEN 894

Whey Protein Isolate

P422.01.2000

ALACEN 894 is a soluble, >90%db whey protein isolate (WPI) product, manufactured by ultrafiltration (UF) and cross flow microfiltration (MF). Microfiltration naturally isolates undenatured protein providing a MF WPI product which is low in fat and high in protein.

ALACEN 894 has excellent nutritive value with True Protein Digestibility of 99.7%, PD-CAAS=1%, and PER=3.0. These qualities make ALACEN 894 particularly useful for protein fortified nutritional foods that require an excellent nutritional profile and/or solubility.

PRODUCT CHARACTERISTICS

- Excellent Nutritive Value
- Clean Flavour
- Soluble Over A Wide pH Range
- Low Fat & Low Cholesterol
- Low Lactose

SUGGESTED USES

- Protein Fortified Nutritional Foods
- Meringue & Whipped Toppings
- Low Fat & Lactose Foods

TYPICAL COMPOSITION ANALYSIS

Protein (N x 6.38) as is	89.9%
Protein (N x 6.38) dry basis	94.1%
Moisture	4.5%
Fat	0.2%
Ash	3.2%
Lactose	0.6%

TYPICAL CHEMICAL ANALYSIS

pH	6.9
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TYPICAL PHYSICAL PROPERTIES

Colour	Cream
Flavour	Clean
Scorched Particles (50g)	Disc A

TYPICAL MICROBIOLOGICAL ANALYSIS

Aerobic Plate Count (cfu/g)	<10,000
Coliforms (/g)	Not Detected
E.coli (/g)	Not Detected
Yeast & Moulds (cfu/g)	<10
Coagulase Positive Staphylococci (/g)	Not Detected
Salmonella (/750g)	Absent
Listeria (/50g)	Absent

TYPICAL MINERAL ANALYSIS

Sodium	280 mg/100g
Potassium	901 mg/100g
Chloride	11 mg/100g
Calcium	310 mg/100g
Phosphorus	199 mg/100g

PACKAGING

The standard pack is a multi-wall paper bag which contains the products within an inner polythene bag. No staples or metallic fasteners are used.

Net weight	20.0kg
Gross weight	20.4kg

STORAGE AND HANDLING

ALACEN 894 should be kept in a cool, dry ventilated place. Temperatures below 25°C, relative humidities below 65% and an odour

free environment will extend storage life.

Packages should not be in contact with walls or floors. Stock should be used in rotation, preferably within 24 months of manufacture.

QUALITY ASSURANCE

Strict quality control procedures are enforced during manufacture. The manufacturing environment is also subject to regular monitoring and control.

Final product is sampled and tested for chemical, sensory and microbial parameters using internationally recognised procedures.

During storage and shipment, precautions are taken to ensure that product quality is maintained. Each package is identified, enabling traceback.

ALATAL 819

Whey Protein Hydrolysate

WPH 926 is a high quality hydrolysate produced by a controlled enzyme treatment of whey protein which provides amino acids, peptides, and polypeptides. The whey protein base material is highly nutritious. Since the enzyme treatment is a mild and carefully monitored process, the essential amino acids remain intact and the high nutritional quality of the original protein is retained. WPH 926 is designed to provide a 10^{-4} -fold reduction in antigenicity while still retaining clean flavor.

TYPICAL COMPOSITIONAL ANALYSIS

Protein (N x 6.38), dry basis %	84.5
as is %	80.9
Ash %	4.7
Moisture %	4.3
Fat %	4.0
Lactose %	3.2
Antibiotics (IU/g)	<0.01

TYPICAL MINERAL ANALYSIS

Sodium %	0.6
Calcium %	0.6
Potassium %	1.0
Phosphorus %	0.3
Magnesium %	0.02
Chloride %	0.3

SUGGESTED USES

- Enteral products for malabsorption
- Lactose-free nutritional products

PRODUCT CHARACTERISTICS

- Excellent nutritional quality
- Reduced allergenicity
- Very good solubility
- Good heat stability
- Low flavor profile

TYPICAL MOLECULAR ANALYSIS

Amino nitrogen %	1.7
Total nitrogen %	13.4
AN/TN %	23
Degree of hydrolysis %	10

RECOMMENDED LABELING

Whey Protein Hydrolysate

TYPICAL MICROBIOLOGICAL ANALYSIS

Standard plate count (cfu/g)	<1,000
Coliforms (cfu/g)	<10
E. coli (1g)	Negative
Yeast & Mold (cfu/g)	<50
Staph. Coag. Pos. (1g)	Negative
Salmonella (750g)	Negative

TYPICAL PHYSICAL PROPERTIES

Color	Cream
Sensory	Clean, slightly bitter
Bulk density (packed, g/mL)	0.50
pH (5% at 20°C)	7.2
Solubility %	100

PACKAGING

Heat sealed, multiwall kraft paper bags. Polyethylene bag liner individually closed. No staples or metallic fasteners.

Net wt.	50.0 lbs. (22.7 kg)
Gross wt.	50.8 lbs. (23.1 kg)

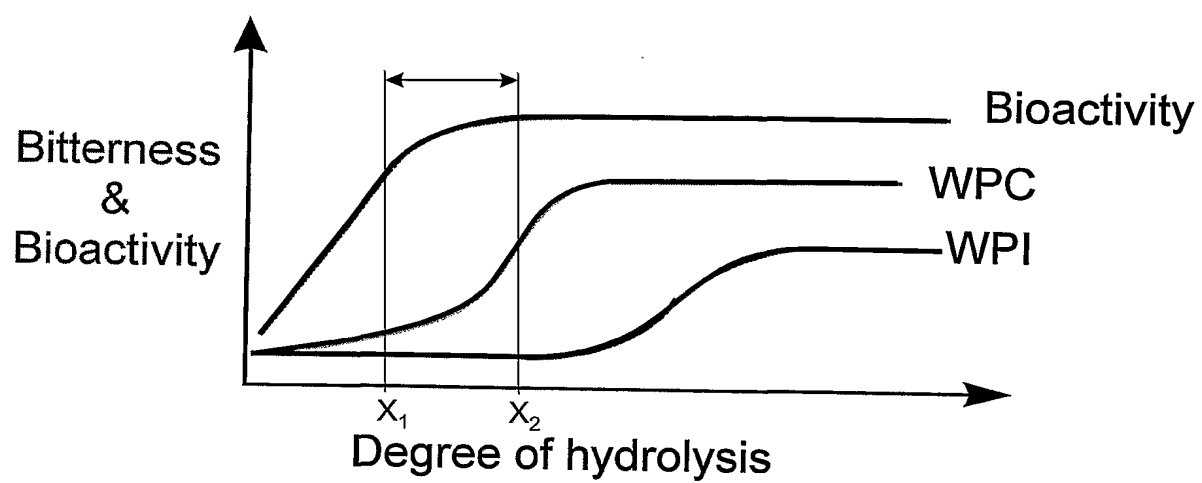
ENZYMES

All protease enzymes used in WPH 926 manufacture are food grade and acknowledged by the U.S. Food and Drug Administration to be Generally Recognized As Safe (GRAS).

STORAGE

Whey Protein Hydrolysates are best used within one (1) year of receipt by customer when stored under proper conditions. Since hydrolysates are hygroscopic and can absorb odors, it is recommended that product be stored at temperatures below 25°C, relative humidity below 65%, and in an odor-free environment.

1 / 2

**Figure 1**

2 / 2

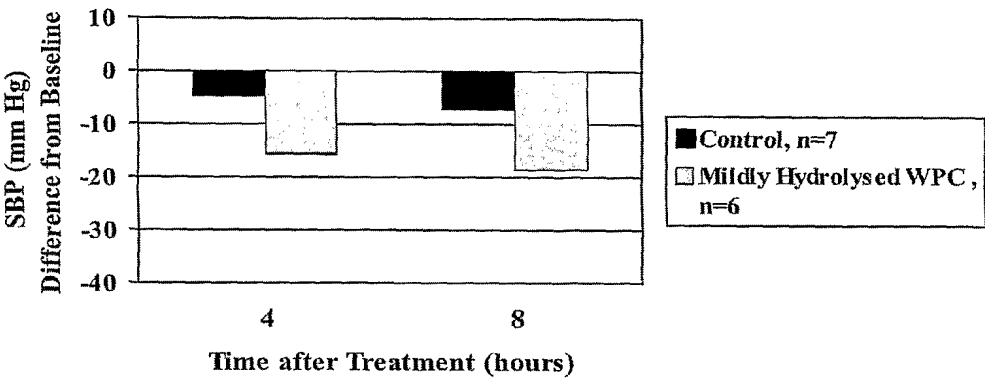


Figure 2

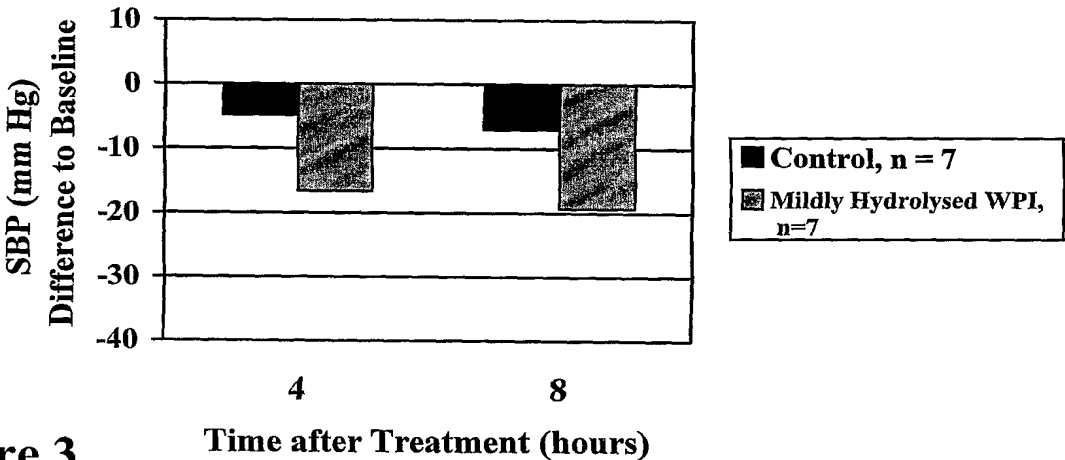


Figure 3

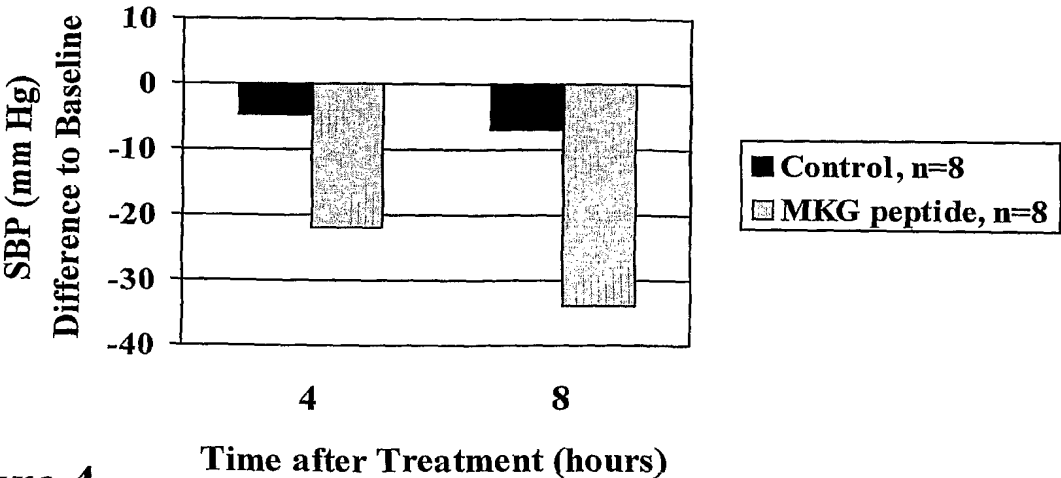


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00188

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: A23J 3/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

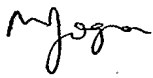
STN (WPIDS;FSTA;CA):whey protein hydrolysate; SEQ ID NOS 1-8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO 9965326, A1 (New Zealand Dairy Board) 23.12.99. See p3, Table 3; claims	1-34
X,Y	EP 269593, B1 (Albuglobe Aktiebolag) 04.03.92. See pp 1-3; Examples; claims	1-14;
X	WO 9221248, A1 (Danmark Protein A/S) (10.12.92). see p1; claims	1-14

☒ Further documents are listed in the continuation of Box C
 ☒ See patent family annex

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 3 December 2001	Date of mailing of the international search report - 7 DEC 2001
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929	Authorized officer  MADHU K. JOGIA Telephone No : (02) 6283 2512

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00188

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9324020, A1 (Novo Nordisk A/S) 09.12.93. See p 2; claims	1-14
X	US 5952193, A (Shimamura et al) 14.09.99; page 14; Examples; claims	1-14
X	WO 9304593, A1 (Teagasc, The Agriculture and food Development Authority) 18.03.93. See p 22; Examples; claims	1-14
Y	WO 9113554, A1 (Novo Nordisk A/S) 19.09.91.	1-14
X	JP 10033115 A (Nouchikusangiyon Shinko Jigyodan; Zenkoku Nokyo Nyugyo Plant Kyokai); abstract	1-14
X	Int Dairy J 6, pp 285-94 (1996) Castro et al "Proteolysis of whey proteins by a <i>Bacillus subtilis</i> enzyme preparation"	1-14

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ01/00188

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9965326	AU	45359/99	EP	1087668	NO	20006305
EP	269593	AU	81278/87	CA	1334635	DK	6168/87
		FI	875192	JP	63160555	NO	874899
		NZ	222592	SE	8605045	US	4847096
WO	9221248	AU	18827/92	CA	2109584	EP	588841
		IE	921762	IL	102059	NO	934310
		NZ	242963	US	5691165		
WO	9324020	AU	43099/93	EP	642307	NZ	253019
US	5952193	AU	36738/95	AU	63603/98	EP	799577
		JP	8112063	NZ	294046	WO	9611584
		JP	8112064	JP	8140693		
WO	9304593	AU	24682/92	EP	604467	IE	913057
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WO	9113554	EP	518999	US	5866357	EP	647276
		US	5523237	WO	9213964	AU	26799/97
		BG	102948	BR	9708859	CZ	9803408
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END OF ANNEX							